Color-tuned Channelrhodopsins for Multiwavelength Optogenetics

Matthias Prigge†‡, Franziska Schneider†§, Satoshi P. Tsunoda†, Carrie Shilyansky§, Jonas Wietek‡, Karl Deisseroth§, and Peter Hegemann‡,2

‡From Institute of Biology, Experimental Biophysics, Humboldt-Universität zu Berlin, 10115 Berlin, Germany
§From Departments of Bioengineering and Psychiatry Howard Hughes Medical Institute, CNC Program, Stanford University, Stanford, CA 94305, USA

1These authors contributed equally to this work
2To whom correspondence may be addressed: hegemape@rz.hu-berlin.de, phone: 0049 30 2093 8681

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Background: Dual-color activation of two cell types with Channelrhodopsins is a major challenge since all available variants absorb blue light.

Results: We engineered Channelrhodopsin hybrids with color shifted spectra, as well as altered kinetics and selectivity.

Conclusions: The results provide deeper insight into Channelrhodopsin function.

Significance: The combination of novel and established Channelrhodopsins can activate distinct cell populations by dual-color excitation.

SUMMARY
Channelrhodopsin-2 is a light-gated ion channel and a major tool of optogenetics. It is used to control neuronal activity via blue light. Here we describe the construction of color-tuned high-efficiency channelrhodopsins (ChRs), based on chimeras of Chlamydomonas Channelrhodopsin-1 and Volvox Channelrhodopsin-1. These variants show superb expression and plasma membrane integration, resulting in 3-fold larger photocurrents in HEK-cells compared to channelrhodopsin-2. Further molecular engineering gave rise to chimeric variants with absorption maxima ranging from 526 to 545 nm, dovetailing well with maxima of channelrhodopsin-2 derivatives ranging from 461 to 492 nm. Additional kinetic fine-tuning led to derivatives in which the lifetimes of the open state range from 19 ms to 5 s. Finally, combining green with blue-absorbing variants allowed independent activation of two distinct neural cell populations at 560 and 405 nm. This novel panel of channelrhodopsin variants may serve as an important toolkit element for dual-color cell stimulation in neural circuits.

INTRODUCTION
Channelrhodopsins (ChRs) are photoreceptors that control photo-movement in green algae. ChRs are small monomeric ion channels (1) that can be functionally expressed in defined cell populations in living animals ranging from nematodes, fruit flies, mice, zebrafish to primates (2-5). ChR has also generated hope for clinical applications such as for recovery of rudimentary vision in blind patients (retinal prosthesis), deep brain stimulation for treatment of Parkinson’s disease, treatment of heart failure by light-induced pacing, peripheral nerve stimulation, and modulation of fear learning (6-10).

Upon light stimulation, ChR conductance comprises an initial current, $I_0$, which decays to a steady-state current, $I_S$. Relaxation from $I_0$ to $I_S$ is commonly called inactivation of the conductance, although these kinetics are in fact the result of an early and a late conducting state (O1 and O2) within the ChR photocycle (11). Correspondingly, the off-kinetics comprises several exponentials in principle, but are frequently well described at physiological conditions by one exponential (2). Despite the wide application of ChR, the use of channelrhodopsin-2 (C2) bears several limitations that often prevent sufficient depolarisation in optogenetic studies. These are for example low expression levels, small unitary conductance, inappropriate kinetics, partial inactivation, and inappropriate ion selectivity. Moreover, simultaneous appli-
culation of the actuator C2 and fluorescent sensors or separate activation of two cell types with different ChRs are still a challenge. In several approaches ChRs have been tailored for modulated absorption, kinetics, and membrane targeting. Owing to the fact that the retinal-binding pocket is conserved in all microbial rhodopsins, we first modified amino acids that are in close contact with the retinal. Replacement of the active site residue E123 by Thr and Ala (ChETA variants) caused faster channel closing, eliminated the voltage sensitivity of the temporal kinetics, and induced a 20 nm bathochromic shift (12). In contrast, substitution of C128 by Ser, or of D156 by Ala or combination of both lead to an extreme extension of the open state lifetime with values up to 30 minutes and allowed on/off switching with dual wavelength light protocols (13-15). Mutation of E90, E123, L132, or H134 does not change photocycle kinetics but alters ion selectivity in favor of H+, Na+ or Ca2+, or reduces inactivation after light step-up or multiple light flashes, respectively (16,17). A helix swapping approach combining structural elements from Channelrhodopsin-1 (C1) and C2 led to improved ChRs with larger currents, absorption maxima around 500 nm and reduced inactivation (18). Moreover, screening of a genomic data-base lead to the identification of Volvox ChR1 in the following named V1 with an absorption maximum at 535 nm (19,20). When expressed in pyramidal neurons, V1 could evoke action potentials with 590 nm light, but widespread use was hampered by low expression in most neuronal cell types. Up to now, experiments in which distinct neuronal populations were separately controlled by dual-color activation are rare but have been demonstrated for the depolarizing C2 in combination with the hyperpolarizing red-absorbing chloride pump halorhodopsin from N. pharaonis (NpHR) (21). Dual-color depolarization experiments require multiple sets of ChR variants with spectrally well-separated absorption, large photocurrents, and perhaps even different operational light sensitivities. Operational light sensitivity is defined by the light power necessary to evoke action potentials in neurons and depends on ChR expression, kinetics, ion selectivity, and conductance, as well as on endogenous cellular characteristics. Operational light sensitivity also subsumes the intrinsic light sensitivity of the actuator in charge, which however is quite invariant for all microbial rhodopsins. Advanced optogenetics will not only depend on actuator probes, but also on the simultaneous use of reporter proteins as genetically encoded fluorescent calcium indicators (GECI) or voltage-sensors (VSFP). To meet this challenge, we implemented a systematic molecular engineering approach, integrating helix swapping as global rearrangement of structural elements with subsequent mutagenesis resulting in local conformational changes or alteration of the hydrogen bond network. This allows us to present a broader color palette of ChRs to effectively fill the gap. The best candidates were tested in hippocampal neurons to validate usability in different cell types. The recently described 3D structure of a distinct C1C2 chimera allows to interpret our results on a molecular level (22).

Experimental procedures
Molecular Biology – Chimeric ChR variants were generated from synthetic human codon-adapted cDNAs of Chr-encoding genes named in the algal data basis Chlamydomonas opsins (COP): COP3, COP4 encoding ChR1 (C1) and CHR2 (C2) (GenBank EU714030.1), and the volvopsins VOP3 and VOP4 encoding VChR1 (V1) and VChR2 (V2). DNA fragments were combined by overlap extension PCR as described elsewhere (18). Resulting PCR fragments were cloned in-frame into pECPF-N1 using XbaI/BamHI. Junction sites of chimeras are summarized in supplemental Table 2. Point mutations were generated with Quikchange (Agilent Technologies, Palo Alto, CA). For oocyte measurements chimera were subcloned into the pGEMHE vector with BamHI/BsiWI. A full-length cRNA was synthesized in vitro from NheI-linearized pGEMHE plasmid using T7 RNA polymerase (Message Machine; LifeTechnologies, Darmstadt, Germany).

HEK Cell Recording – HEK293 cells were cultured as described (24) and seeded onto coverslips at a concentration of 0.175 x 10⁶ cells · ml⁻¹ and supplemented with 1 µM all-trans-retinal. Transient transfection was performed using Fugene 6 (Roche, Mannheim, Germany) 20 – 28 h before measurements. For selectivity measurements a cell line stably expressing C2-mVenus was used (14). Signals were amplified and digitized using AxoPatch200B or Heka EPC7 and DigiData1400. For recording wavelength dependency a light guide from a Polychrome V unit (TILLPhotonics, Planegg, Germany) was mounted at the epif-illumination port of an Olympus IX70 microscope resulting in a final light intensity between 0.05 – 0.23 mW
3 mm plastic light guide also mounted into the epi-illumination path of the microscope and combined with the beam from the polychrome unit via a 70 % R / 30 % T beam splitter. Combined fura-2 and C2 excitation light was guided to the objective via a dualband dichroic mirror (FF493/574, AF-Analysetechnik, Tübingen, Germany). This allowed us to excite fura-2 at 380 nm and monitor fluorescence between 493 and 535 nm. Using the same filter, C2 could be excited at 405, 470 and 560 nm with 0.3, 0.8 and 0.17 mW · mm⁻² for 405, 450 and 560 nm, respectively. Fura-2 excitation was reduced to 0.005 mW · mm⁻².

For measurements of light-induced opening of Caᵥ3.2 (Fig. 3C) recordings were performed at the following ion composition [in mM]: 140 NaCl, 2 KCl, 2 MgCl₂, 2 CaCl₂ and 10 Hepes. pH was adjusted to 7.2 with NMG/HCl. Fura-2 fluorescence was recorded at an exposure time of 500 ms at 380 nm and a sampling rate of 0.3 Hz. ChRs were excited for 100 ms with the indicated wavelengths.

For experiments testing calcium selectivity of different ChR variants (Fig. 3G), cells were measured in the following buffer [in mM]: 5 NaCl, 1 KCl, 2 MgCl₂, 10 Hepes and 70 CaCl₂. Fura-2 fluorescence was recorded at 380 nm with an exposure time of 500 ms and a sampling rate of 0.3 Hz. ChRs were excited with their corresponding wavelength for 10 s. ΔF over F₀ was calculated as given in Tian et al. (25).

**Oocyte Photostimulation and Recording – Xenopus oocytes** (Ecocyte Bioscence, Castrop-Rauxel, Germany) were injected with 50 nl ChR cRNA (0.5 to 1 µg · µL⁻¹) and incubated in the dark at 18°C in Ringers solution for 3 – 5 days. Two-electrode voltage clamp measurements were performed on Xenopus oocytes using a Turbo Tec-05X (NPI Electronic) and a DigiData 1440A interface. For fast kinetic analysis, cells were stimulated with a tuneable 7-ns laser as described (19).

**Electrophysiology in Hippocampal and Cortical Neurons** – Primary cultured hippocampal neurons were prepared from P0 Sprague-Dawley rat pups (Charles River). CA1 and CA3 were isolated, digested with 0.4 mg · ml⁻¹ papain ( Worthington), and plated onto glass coverslips precoated with 1:30 Matrigel (Becton Dickinson Labware), and grown on coverslips in a 24-well plate at a density of 65,000 cells per well. DNA was expressed in hippocampal pyramidal neurons in vitro. DNA maps and clones are available at [http://www.optogenetics.org](http://www.optogenetics.org). Transfections were carried out 6 - 10 days post culturing. For each well, a buffered DNA-CaCl₂ mix...
was prepared and incubated at room temperature for 20 min. The mix was added dropwise into each well (from which the growth medium had been removed and replaced with prewarmed minimal essential medium (MEM)) and transfection proceeded for 45-60 min at 37 °C, after which each well was washed and the original growth medium was returned. Standard electrophysiology recordings were carried out on day 4 - 6 after transfection.

Recordings from individual neurons identified by fluorescent protein expression were obtained in Tyrode media ([mM] 150 NaCl, 4 KCl, 2 MgCl₂, 10 D-glucose, 10 HEPES, 0.001 TTX, pH 7.35 with NaOH) using a standard internal solution ([mM] 130 KGlucanote, 10 KCl, 10 HEPES, 10 EGTA, 2 MgCl₂, pH 7.3 with KOH) in 3 - 5 MΩ glass pipettes. Rhodopsins were activated by color filtered light from a Xenon lamp source (Sutter Instrument DG-4) that was attenuated by neutral density filters. A minimum of 30 s delay was imposed between stimulus sweeps to minimize confounding effects of inactivation.

Confocal microscopy – Cells were imaged 1 day post-transfection. Pictures were taken on a confocal Olympus LSM IX81 equipped with a 60 x 1.2 Water UplanSApo objective. 440 and 561 nm LEDs were used for excitation. Fluorescence emission was detected using a spectral PMT detector.

Data analysis – Data was analyzed using TillVision Software or pClamp10.1 and further processed by SigmaPlot and Adobe illustrator. Standard errors are given for all results. Initial currents I₀ were determined by linear extrapolation to t=0. Iᵢ reflects average current after 300 ms of light activation. Action spectra were linearly normalized on the light intensity at each wavelength.

RESULTS
To engineer a highly-expressing ChR with large photocurrents and absorption red-shifted relative to ChR2 (C2), we started with VChR1 (V1) since this protein exhibited the most red-shifted absorption spectrum of all known ChRs at the beginning of the project (Fig. 1C dark grey). The spectral broadening is based on the simultaneous occurrence of the alkaline and acidic isoforms at neutral, external pH, (20). Since V1 is only poorly targeted to the membrane in mammalian host cells (Fig. 1D), we followed the strategy of Yawo and colleagues by exchanging helices of V1 with homologous helices of related ChRs (18). In a first set of experiments we systematically replaced helices of V1 by helices of the highly expressing Volvox VChR2 (V2, Fig. 1A, second row). Exchange of helices H3 and H4, H2 to H4, or H5 and H6 resulted in hybrids with 1.5 fold increased photocurrents in HEK cells and V1-like pH₀-dependent absorption spectra still overlapping at neutral pH₀. Interestingly, chimeras with H6 and H7 of V2 (V1V2-52 and V1V2-25) expressed well and exhibited large photocurrents, but the spectra were blue-shifted to 470 nm (Fig. 1A). For further improvement, we tested constructs employing N-terminal helices of either V2 or C1 (Fig. 1A, third row). Chimeras V2V1-25, V2V1-43 and V2V1-52 were non-functional in HEK cells. V2V1-61 was functional, but without any improvement over V1. Since several C1C2 chimeras had shown good photocurrents in previous studies we constructed chimeras with N-terminal helices of C1 (18). C1V1-52 integrated exceptionally well into the membrane but photoconductance was still small and in the range of wt V1. Finally, we restricted the C1 part to the first two helices, which still resulted in a variant with excellent membrane integration (supplemental Fig. 1A). This, so called, C1V1-25 variant, was originally prepared as two variants, namely C1V1-25A with the first extracellular loop (ECL1) of C1 and C1V1-25B with ECL1 of V1 (supplemental Fig. 1A). For the sake of simplicity, C1V1-25A will be named C1V1 in the following text. Both constructs were again well targeted to the membrane but the B variant elicited larger photocurrents beyond 2000 pA (Fig. 3D, dark grey). We have previously reported on aspects of these constructs in the context of neuronal application (15); most of the mutants reported here are based on the original C1V1-25A. For color tuning of C1V1 we replaced E122 for Thr (E83 in C2) in helix H2, a residue that is part of the inner gate and in indirect contact with the chromophore (22). This mutant C1V1-ET exhibited large photocurrents with only moderate inactivation and excellent membrane targeting (Fig. 1D and supplemental Table 1). The spectrum showed an additional red shift to λ max = 545 nm (Fig. 1C, orange). To fill the spectral gap between the current-improved C2-variant C2-ET-TC (E123T-T159C) with peak absorption near 495 nm (23), and C1V1-ET (E122T), (Fig. 1C dark blue and orange) we exchanged in C1V1 the polar Ser220 near the β-ionone

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1 This annotation means that H1 - H5 originate from V1, H6 and H7 from V2
ring by an unpolar Gly (see Fig. 2A for position). The resulting C1V1-S220G (C1V1-SG) retained its large photocurrent (Fig. 1B, green) and the spectrum was 13 nm blue-shifted towards 526 nm (Fig. 1C).

In Fig. 1E and F representative current traces for light pulses and laser flashes are shown for the well expressing color variants. The C1V1-SG and C1V1-ET off-kinetics in flash experiments (τ ≈ 117 and 73 ms) were close to the value for C1V1 (τ ≈ 77 ms). But, recovery of the peak current was fast in the case of C1V1-ET (τ ≈ 2.5 s) and slow in the case of C1V1-SG (τ ≈ 11 s) (Fig. 1G, yellow and green) compared to unmodified C1V1 (τ ≈ 4 s). Except for C2-ET-TC, the recovery of the initial current I₀ in C2 and all C1V1-derivatives is slower at more positive voltage (supplemental Fig. 1D,F). To change the open-state lifetime of the new color variants, we extended the mutational study with a focus on slow-cycling ChRs (Fig. 2A). Replacement of D195 by Ala in C1V1 (D156A in C2) lead to a reduction of the current size without any slow-down of the photocycle (τ_{off} ≈ 86 ms), whereas Ser at position C167 (C128S in C2) caused a 40-fold larger τ_{off} (~4 s) (supplemental Table 1). The slowdown of C167S is still 25 times smaller compared to the effect in the corresponding C2 mutation and it was conceivable that the insensitivity of the photocycle kinetics is caused by the neighbouring C198, which is Thr in C2 (T159). We tested this possibility in C2 by introducing T159C on the C2-C128S background in C2, which caused current enlargement with acceleration of the photocycle with an open state life time of 4 s compared to 106 s in support of our hypothesis (supplemental Fig. 3B). To produce color variants with slow kinetics, the color tuned C1V1-ET (E122T) and C1V1-SG were combined with the C167S mutation (Fig. 2A). The double mutants, C1V1-ET-CS and C1V1-CS-SG, showed small photocurrents and off-kinetics between 1.5 and 4.5 s (Fig. 2B).

The current reduction is most readily explained by a strong overlap of the red-shifted dark state with the conducting state (P520 in C2), which causes immediate photochemical back reaction in continuous light at all wavelengths between 520 and 580 nm. Moreover, this overlap of spectra also precludes optical back conversion by a red-shifted light stimulus (supplemental Fig. 1B) after the actinic light is switched off. In contrary, the red-shifted dark state is facilitating photochemical back reaction at 400 nm light, which is more complete for C1V1-based than C2-based slow-cycling ChRs. The reason is that the photocycle intermediate P390, which is in equilibrium with the conducting state (14,15), is spectrally more separated from the dark state compared to C2-C128S.

For generation of fast-cycling C1V1 mutants we tested point mutations that had caused photocycle acceleration in other ChRs. Positions of successfully mutated residues are again shown in Fig. 2A. The E162T exchange (E123T in C2) resulted in 3 fold faster off-kinetics and a 20 nm blue-shifted spectrum with some broadening of the spectrum as already published (15). The blue shift was unexpected since the corresponding ChETA-mutation in C2 caused a 20 nm red shift (12).

Interestingly, combining E162T with E122T (C1V1-ET-ET) prevented the spectral broadening and showed a faster τ_{off} ≈ 15 ms (supplemental Table 1). Since the maximal absorption of C1V1-ET-ET peaks at 528 nm, it is an alternative for C1V1-SG whenever fast repetitive green light stimulation is required. Prior to the detailed biophysical characterization we already have employed this variant of our screen for the manipulation of Neocortical excitation/inhibition balance (15). To further accelerate deactivation kinetics, C1V1-ET was combined with V196I and G197A mutations in H4 (157 & 158 in C2) (Fig. 2A) (22), which were expected to distort the functionality of C198 due to subtle structural changes. When introduced separately, the off-kinetics showed 123 and 79 ms relaxations, whereas the combination C1V1-ET-VI-GA (C1V1-triple) caused faster off-kinetics of ≈ 41 ms, respectively with currents still larger than C2 currents (Fig. 2C). But, upon 10 Hz stimulation (Fig. 2D) the C1V1-triple performed with 48 % inactivation and significant offset currents slightly better than C2-TC (60% inactivation) (supplemental Table 1), while under-performing relative to C2-ET-TC and C1V1-ET-ET that showed less inactivation and smaller offset currents.

To compare the real efficiency of light activation (intrinsic light sensitivity) for different ChRs in host cells, we recorded light titration curves for the early peak current I_p and the stationary current I_s for slow and fast cycling variants upon stimulation with 7 ns flashes and 5 s light pulses (Fig. 2E,F). These experiments showed that the efficiency of the primary photoreaction is identical in wt C2, C1V1 and step-function ChRs, although for the latter the rise time of the photocurrents is much slower (Fig. 2E inset). In contrast, the light sensitivity of the steady state currents I_s is a function of light absorption, lifetime of
the open state and photochemical inactivation. Consequently, step-function currents saturate at much lower steady state light intensities (Fig. 2F).

We next tested wavelength-dependent spiking performance with parallel transduction of C2-LC-TC, a high conductance C2 variants discussed below, and C1V1-ET-ET in cultured hippocampal neurons (23). Spike trains were evoked by light stimulation using 3 ms pulses at frequencies of 20 Hz. Figure G and H show the fraction of successful spikes at the indicated wavelengths and light power densities in cells expressing C2-LC-TC or C1V1-ET-ET.

Spike elicitation at 20 Hz by 560 nm was found to confer specificity between both variants across light intensities. For 405 nm light at lower intensities it was also possible to separate groups, 470 nm for C2-LC-TC and 542 nm for C2-TC or C1V1-ET-ET. Based on these results we employed a fura-2 based Ca²⁺ assay in a cell line that stably expresses a mTrek potassium channel and a voltage-gated Ca²⁺-channel (Caᵥ3.2) (23). At given cytoplasmic ion concentrations, the steady state membrane voltage in darkness is controlled by extracellular potassium, and depolarisation can be modulated with light via C2-TC or, alternatively, C1V1-triple. At sufficient depolarisation the Caᵥ3.2 channels open and Ca²⁺ influx is monitored as fura-2 fluorescence as shown in the pictogram in Fig. 3C (24).

For activation of two HEK cell populations with different wavelengths we compared responses of C1V1, C1V1-triple, and C2-TC at 405 or 560 nm light (Fig. 3A,B). C1V1-triple shows full activation at 560 nm and residual activation at 405 nm that is clearly smaller than for C1V1 (grey). In contrast, the reaction of C2-TC is half maximal at 405 nm with zero cross reactivity at 560 nm. The 2-fold larger peak photocurrent of C2-TC compared to C2 provides a significant advantage compared to C1V1 provides a significant advantage.

For stimulation at 405 nm. Based on these results we employed a fura-2 based Ca²⁺ assay in cell line that stably expresses a mTrek potassium channel and a voltage-gated Ca²⁺-channel (Caᵥ3.2) (23). At given cytoplasmic ion concentrations, the steady state membrane voltage in darkness is controlled by extracellular potassium, and depolarisation can be modulated with light via C2-TC or, alternatively, C1V1-triple. At sufficient depolarisation the Caᵥ3.2 channels open and Ca²⁺ influx is monitored as fura-2 fluorescence as shown in the pictogram in Fig. 3C (24). This assay allowed us to directly compare the performance of blue and green absorbing ChRs for membrane depolarisation. The cells displayed in Fig. 3C express C1V1-triple-eCFP and C2-TC-mCherry seen as cyan and red fluorescence. Excitation with 405 nm light caused a rapid decrease in fura-2 fluorescence in cells expressing C2-TC whereas 560 nm light evoked Ca²⁺ influx in the C1V1-triple expressing cells only. Thus the 405 nm and 560 nm light were almost perfectly selective. In contrary, 470 nm light excited both C2-TC and C1V1 equally well causing Caᵥ3.2-mediated Ca²⁺ influx in both cell types. Light power at 405 nm of up to 0.03 mW · mm⁻² for C2-TC excitation was used without cross-activating C1V1.

Finally, to characterize cation flux through ChRs we recorded photocurrents at various ionic conditions. We included in this survey C2-L132C (C2-LC) for which an enhanced Ca²⁺ selectivity had been reported (17). We introduced the analogous mutation into C1V1, but L171C was only weakly expressed. It was conceivable that L171C is destabilized by interaction with the Cys at position 198 (T159 in C2 as shown in supplemental Fig. 3). However, the C2 double mutant C2-L132C-T159C (C2-LC-TC) showed superb performance (Fig. 3D). We also included in the survey both C1V1 variants, A and B. Total peak currents under standard conditions are compared in Fig. 3D revealing that C1V1-B, C2-LC, C2-TC and C2-LC-TC mediate almost threefold larger peak currents than wt C2, whereas C1V1-A currents were clearly smaller. Photocurrents of C2-LC-TC were most robust and consistent. Peak currents were normalized to the levels at pH₀ 7.2 and 140 mM Na⁺. Different grades of inactivation can be seen from individual traces in Fig 3E. Next, photocurrents were measured at pH 9 and were compared to I₀ of pHₙ 7.2. (Fig. 3F). At 2 mM Ca²⁺ and 2 mM Mg²⁺ conductances were small as expected, but the Iₛ was significantly larger for C2-LC and C2-LC-TC. At 140 mM Na⁺ the I₀ was only slightly enlarged for C1V1, C2-LC, C2-TC and C2-LC-TC compared to wt C2. But, this increase was even more obvious at high Ca²⁺, suggesting that all 4 variants have particularly enhanced Ca²⁺-selectivity (Fig. 3F upper panel and supplementary Fig. 2A). Notably, for Ca²⁺-influx in continuous light the stationary current Iₛ is of much higher relevance than the peak currents I₀. Our experiments show that Iₛ under high Ca²⁺ is large for C1V1, C2-LC and C2-LC-TC, but is small for C2-TC and wt C2 (Fig. 3F lower panel and supplemental Fig. 2B red traces). In summary, at -60 mV and symmetric pH 9, C2-LC-TC shows 7.4 fold larger Ca²⁺ peak currents (Iₛ), and 19 times larger Iₛ compared to C2 (Fig. 3D-F) taking the normalization...
into account (supplemental Table 2). C1V1-B shows 7.1 fold larger \( I_0 \) and 10 fold larger \( I_S \) under similar conditions. However, this enhancement might be quite different at higher voltage (less negative) and low pH. Since Ca\(^{2+}\) mediated currents are superimposed by proton currents at neutral pH, the Ca\(^{2+}\) fraction of the inward current is difficult to distill from electrical recordings at neutral pH, (Fig. 4D,E). Thus, we visualized the Ca\(^{2+}\) influx again by a fura-2 based Ca\(^{2+}\) assay (Fig. 3G). Whereas C1V1-A showed only slightly higher Ca\(^{2+}\) influx because of its only moderately improved expression, C1V1-B delivered significantly higher Ca\(^{2+}\) influx than C2-TC and C1V1-A due to its higher expression level. However, the fura-2 assay also revealed that C2-LC and particularly C2-LC-TC under neutral conditions promote higher Ca\(^{2+}\) influx in continuous light, under conditions of competition with H\(^{+}\).

**DISCUSSION**

We applied a helix exchange strategy to *Volvox* V1 and revealed a well-expressing C1V1-chimera with a 70 nm red-shifted absorption relative to the commonly used C2. We also showed that the earlier reported kinetic tuning of C2 can be transferred to the new C1V1 chimera, although the effects of individual mutations differ quantitatively and substantially from related mutations in C2. By a systematic exchange of V1 helices by V2 homologues we identified the helices H6 and H7 as the most essential for the absorption difference between V1 (535 nm) and V2 (465 nm), and helices H1 and H2 as most critical for good expression. Certainly, it was conceivable that the 39 extra amino acids in the N-terminal region of C1 contain sequences or even single amino acids that are responsible for better membrane integration or folding, although both of the parental V1 and C1 rhodopsins are poorly expressed. Therefore we suggest that interaction between helix H1 and H7 is the most relevant parameter for the excellent membrane integration of C1V1, also in accordance with the C1C2 structure (22). A light-dependent modulation of the tilting angle between H1 and H7 could be a molecular mechanism for opening and closing of the ion channel pore formed by H1, H2, H6 and H7 (22). To test this hypothesis an open state crystal structure is mandatory. Nevertheless, even small differences in the ECL1 loop region, as between C1V1-A and B, can have variable effects on the expression level in different host systems. Moreover, the natural presence of Cys at position 198 (T159 in C2) is an additional factor for effective membrane integration as shown by the large C2-TC currents and the small currents of the C1V1-C198T. As noted above, the mutation E122T in C1V1 (E83 in C2) showed an extra 5 nm bathochromic shift to 545 nm rendering it the most red-shifted ChR so far. Rather unexpected was the blue shift of E162T, a mutation that caused a red-shift in C2 (E123T in C2) (12). Thus the arrangement of counterion of the protonated retinal Schiff base must be different in C1V1 compared to C2, probably allowing anions such as Cl\(^{-}\) to replace the Glu in the case of C1V1-ET but not in C2-ET. Any approach to shift the C1V1-ET absorption further in the bathochromic directions by single mutations was unsuccessful; further color tuning will therefore likely rely on several subtle amino acids exchanges, which then synergistically shift absorption. This remains a difficult task even with a high-resolution structure in hand because the counterion arrangement remains difficult to modify. A promising alternative is complementary mining of genomic databases of other algae or other lower eukaryotes for natural red-shifted ChRs that can in turn be further modified according to the strategy outlined here. Since modification of D195 in C1V1 did not result in a slower photocycle (in contrast to similar changes of the homologous residue D156 in C2), we can effectively rule out the direct interaction of D195 with C167 (D156-C128 in C2) as proposed by Bamann et al. for C2 (14) (see supplemental Fig. 3A). It is also intriguing that C2 based slow ChRs can be switched off with 405 and 590 nm light, whereas C1V1-based versions are only efficiently inactivated by 405 nm. As we proposed above, this observation can be elegantly explained by a close overlap of the dark state spectrum with the spectrum of the conducting state due to a smaller absorption shift of the latter. This would also explain, at least partially, why the maximal currents of the C1V1-based step-function tools are small. In Fig. 3 we show that the early Ca\(^{2+}\) current \( I_0 \) of C1V1 is larger than \( I_0 \) of C2 and about equal to \( I_0 \) of C2-LC at pH\(_0\) 9, whereas the stationary C1V1 Ca\(^{2+}\) current is twofold smaller compared to C2-LC or C2-LC-TC. However, Ca\(^{2+}\) competes with other ions as we recently evaluated quantitatively for wt C2 and some mutants (16). This competition is quite different for \( I_0 \) and \( I_S \) as seen from the I-\( V \) plots in Fig. 4 and supplemental Fig 2. For C2 the reversal potential \( E_R \) for \( I_0 \) at 70 mM Ca\(^{2+}\) pH\(_0\) 9 and pH\(_i\) 9 is near +45 mV and is near zero at 2 mM Ca\(^{2+}\). This shift correlates
with the Nernst Potential change and suggests that at 70 mM Ca\(^{2+}\) most of the current is carried by Ca\(^{2+}\) and very little Ca\(^{2+}\) is conducted at 2 mM Ca\(^{2+}\) (supplemental Fig. 2A). In contrary, for the stationary current \(I_0\) \(E_r\) is still zero at 70 mM Ca\(^{2+}\) (Fig. 4A) indicating that the contribution of Ca\(^{2+}\) to \(I_0\) is very minor even at high Ca\(^{2+}\). It was puzzling to see that at neutral pH\(_{i}\) the differences in the reversal potentials disappear (Fig. 4D), whereas at neutral internal pH and high external pH H\(^+\) efflux is dominating photocurrents but with a higher influence on \(I_S\) compared to \(I_0\) \(E_r\) for \(I_S\) is more negative than \(E_r\) for \(I_0\) (Fig. 4E). This again supports the idea that internal and external protons compete with Ca\(^{2+}\) especially during the stationary phase. For C1V1 the situation is qualitatively and quantitatively different. At -60 mV, \(E_r\) for \(I_0\) is similar to that of C2 for \(I_S\) and also \(I_0\) has similar \(E_r\), suggesting that the channel selectivity has not changed during adaptation. Similarly, in C2-LC-TC, both \(E_r\) for \(I_0\) and \(I_S\) are again very similar but more positive, indicating that C2-LC-TC is even more Ca\(^{2+}\) selective than C1V1. The greater Ca\(^{2+}\)-influx of C2-LC-TC (and with some reduction of C1V1) as seen in the fura-2 assays is multifactorial, and the twofold higher Ca\(^{2+}\)-selectivity is only one contributory factor. Equally important is the 3-fold better expression, lower inactivation and reduced H\(^+\)-competition. Consistent with these HEK cell measurements, our neuronal experiments revealed an improved performance for C2-LC-TC and C1V1. Both variants provide the cells an improved operational light sensitivity (Fig. 2G) and therefore allowed a better dual-color activation at lower light-intensities compared to previous studies with wild type V1 (20). Surprisingly, C2-LC-TC enabled successful spiking similar to C2 despite its 10 times slower off-kinetics (14) (Fig. 2I), supporting the assumption that higher Ca\(^{2+}\) permeability may facilitate faster repolarisation through activation of secondary channels (17). Since the LC mutation results in non-functional C1V1, we conclude that the best Ca\(^{2+}\)-conducting variant for green and yellow light stimulation remains C1V1-B.


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FOOTNOTE
M.P., FS, P.H. and K.D. designed research, analyzed the data, and wrote the paper; S.T. carried out laser experiments and C.S. experiments in neurons; J.W. installed a computer-controlled light attenuation system.

The authors declare no competing financial interest.

FIGURE LEGENDS
Figure 1
Engineering of channelrhodopsin chimeras with different spectral properties. A. Color-coded ChR
chimeras. Peak photocurrent amplitudes are qualitatively indicated by "+" and peak absorption wavelengths are given numerically. In the cases where two wavelengths numbers are listed they reflect two distinct maxima recorded at pHo 5 and 9, that overlap at pHo 7 and broaden the spectrum. B. Averaged peak photocurrent amplitudes for all high-efficiency color-tuned ChRs and their parental origins in HEK cells. C. Action spectra of color-tuned ChR variants in comparison with action spectra of V1 (dark grey) exhibiting a blue shoulder and C1V1 (light grey) (N = 5, 3, 5, 8, 3 and 3 for C2-HR, C2-ET-TC, V1, C1V1-A, C1V1-SG and C1V1-ET). Amplitudes were linearly corrected to light intensities. D. Confocal images of HEK cells expressing color variants and their parental ChRs. E. and F. Normalized photocurrents upon pulse (in HEK cells) or laser flash stimulation (in Xenopus oocytes) for all color mutants. Photocurrents decay biexponentially after activation for 300 ms whereas off-kinetics of dark-adapted ChRs after laser activation can be fitted by a single exponential. Respective \( \tau_{off} \) values and their relative amplitudes are given numerically. G. Recovery kinetics of the transient peak in a 2-pulse experiment at pHo 7.2 for color mutants. Between pulses membrane voltage was kept at -60 mV (N = 4, 4, 4 and 3 for C1V1-ET, C2-ET-TC, C2-RR and C1V1-SG). A typical photocurrent trace is shown as an inset (light blue).

Figure 2
Generation of fast and slow cycling color-tuned ChRs. A. Structural model of C1V1 based on the 3D structure of C1C2-52 (pdb: 3UG9) (23) depicting all relevant amino acid positions. B. and C. Average peak photocurrent amplitudes of ChRs in HEK cells at their respective peak wavelength for slow and fast cycling mutants (> 10 mW · mm⁻²). D. Normalized current responses to light trains of 10 Hz for fast cycling mutants. e: Stimulus-response curves of peak currents \( I_P \) upon 7 ns laser flashes (N = 5, 3 and 4 for C2-TC, C2-CT and C1V1). The inset shows the respective current traces for C2-TC and C2-CT. F. Light titration curve for \( I_0 \) of slow and fast cycling mutants upon stimulation with 5 s light pulses (N = 5, 3 and 5 for C2-ET-TC, C2-CS and C1V1). Inset exemplifies individual traces for C2-CS and C2-ET-TC. G – I. Action potential firing in hypocampal neurons. G. At 560 nm light C1V1-ET triggers spikes reliably compared to C2-LC-TC. H. At 405 nm light C2-LC-TC is applicable to evoke spikes at 0.875 mW · mm⁻² whereas no spikes are seen for C1V1-ET at similar intensities. In contrast at 8.2 mW · mm⁻² C1V1-ET evokes spikes with same probability as C2-LCTC. I. In responding to trains of light pulses up to 20 Hz, both C2-LC-TC and C1V1-ET are spiking with high fidelity. However at 40 - 50 Hz and above, the probability of successful spike generation is significantly reduced compared to 5 Hz (N > 4) particularly for C1V1-ET-ET.

Figure 3
Dual light excitation and ion selectivity for blue and green light absorbing ChRs. A. Action spectra for C2-TC (blue), C1V1 (grey) and C1V1-triple (orange) (N = 2, 8 and 9). Excitation at 405, 470 and 560 nm is indicated by colored bars. B. Responses of C2-TC, C1V1 and C1V1-triple upon 10 ms light pulses at 405 and 560 nm. C. \( Ca^{2+} \) response in two mixed cell populations. Membrane potential was adjusted through mTrek potassium channel with extracellular K⁺. \( Ca^{2+} \) influx through CaV3.2 was monitored by a change in fura-2 fluorescence. HEK cells were separately transfected with C1V1-triple-eCFP and C2-TC-mCherry as shown as fluorescence and transmission overlay (left) and as fura-2 emission (right). Corresponding fura-2 traces for cyan and red fluorescent cells are shown underneath (2 trials with 8 and 13 cells for C2-TC and C1V1-triple). D. and E. Peak photocurrent amplitudes and profiles for C2, C1V1-A, C1V1-B (both exhibit virtually identical profiles), C2-LC, C2-TC and C2-LC-TC for 300 ms light pulses. F. Average initial currents (upper panel) and stationary photocurrents with standard deviations (below) at different ionic conditions. Intracellular buffer was kept at 110 mM NMG Tris pH 9.0. Currents were normalized to \( I_0 \) at standard conditions. All data points are evaluated at -60 mV (each N > 8). G. Fura-2 fluorescence in ChR-expressing HEK cells after light stimulation for 10 s (black bar) at an extracellular \( CaCl_2 \) concentration of 70 mM at pHo 7.2 (N > 10 cells). Fluorescence intensity before light application is normalized to 1.

Figure 4
Current-voltage relationships for C2, C1V1-A and C2-LC-TC. Photocurrents were recorded at high \( Ca^{2+} \) (70 mM) and variable internal pH (pHi=9 for A – C, pH= 7.2 for D. and E.). Reversal potentials of initial currents \( I_0 \) (filled circles) and stationary currents \( I_s \) (open circles) are indicated by green arrows (N > 8).
Figure 1

A

photocurrent

B

C

D

E

F

G

Recovery of $I_p / I_p_{ref}$

$\tau_{rec}$ (s)

$\Delta t$ / (ms)

- 60 mV

100 ms
Figure 2

A

B

C

D

E

F

G

H

I

Figure 2
Figure 4

A  pH$_i$ 9.0, pH$_o$ 9.0

B  pH$_i$ 9.0, pH$_o$ 9.0

C  pH$_i$ 7.2, pH$_o$ 7.2

D  pH$_i$ 7.2, pH$_o$ 7.2

E  pH$_i$ 7.2, pH$_o$ 9.0

- I$_0$, 70 mM Ca$^{2+}$
- I$_s$, 70 mM Ca$^{2+}$
Color-tuned Channelrhodopsins for Multiwavelength Optogenetics
Matthias Prigge, Franziska Schneider, Satoshi P. Tsunoda, Carrie Shilyansky, Jonas Wietek, Karl Deisseroth and Peter Hegemann

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